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Pernicious effects of long-term, continuous 900-MHz electromagnetic field throughout adolescence on hippocampus morphology, biochemistry and pyramidal neuron numbers in 60-day-old Sprague Dawley male rats☆

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ABSTRACT

The central nervous system (CNS) begins developing in the intrauterine period, a process that continues until adulthood. Contact with chemical substances, drugs or environmental agents such as electromagnetic field (EMF) during adolescence therefore has the potential to disturb the development of the morphological architecture of components of the CNS (such as the hippocampus). The hippocampus is essential to such diverse functions as memory acquisition and integration and spatial maneuvering. EMF can result in severe damage to both the morphology of the hippocampus and its principal functions during adolescence. Although children and adolescents undergo greater exposure to EMF than adults, the information currently available regarding the effects of exposure to EMF during this period is as yet insufficient. This study investigated the 60-day-old male rat hippocampus following exposure to 900 megahertz (MHz) EMF throughout the adolescent period using stereological, histopathological and biochemical analysis techniques. Eighteen male Sprague Dawley rats aged 21 days were assigned into control, sham and EMF groups on a random basis. No procedure was performed on the control group rats. The EMF group (EMFGr) was exposed to a 900-MHz EMF for 1 h daily from beginning to end of adolescence. The sham group rats were held in the EMF cage but were not exposed to EMF. All rats were sacrificed at 60 days of age. Their brains were extracted and halved. The left hemispheres were set aside for biochemical analyses and the right hemispheres were subjected to stereological and histopathological evaluation. Histopathological examination revealed increased numbers of pyknotic neurons with black or dark blue cytoplasm on EMFGr slides stained with cresyl violet. Stereological analyses revealed fewer pyramidal neurons in EMFGr than in the other two groups. Biochemical analyses showed an increase in malondialdehyde and glutathione levels, but a decrease in catalase levels in EMFGr. Our results indicate that oxidative stress-related morphological damage and pyramidal neuron loss may be observed in the rat hippocampus following exposure to 900-MHz EMF throughout the adolescent period.

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1. Introduction

The hippocampus is an essential component of the limbic system and of such diverse functions as memory acquisition and integration and spatial maneuvering (Squire, 2004; Moser et al., 2008; Yau et al., 2015). Long-term memory is created by processing

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new, short-term memories and storing these on a temporary basis before permanent deposition in the cortex (Yau et al., 2015). Studies show that the hippocampus plays a crucial role in this temporary deposition and in the recovery of contextual fear memory for up to 2–3 weeks following the original learning process (Kim and Fanselow, 1992; Yau et al., 2015). Therefore, in addition to compromising the normal morphological architecture of the hippocampus, there is also a strong probability that exposure to long-term drugs, chemical agents or any environmental factor will also impair important functions such as these. Studies have shown that electromagnetic field (EMF) may be one such agent (Bas et al., 2009a,b; Baş et al., 2013).

Previous studies have reported that exposure to EMF may cause numerous changes capable of affecting the normal life cycle, depending on the length of exposure and the intensity of the electrical field, and that these may be seen in the form of morphological (Bas et al., 2009a,b; Baş et al., 2013), physiological or behavioral changes (Salford et al., 2003; Baş et al., 2013; İkinci et al., 2013; Odacı et al., 2013). One of the main sources of EMF capable of causing these changes in humans is the cell phone. Due to their being held close to the head in particular, it has been suggested that EMF originating from cell phones can have an adverse impact on the brain and associated structures (Davis et al., 2013).

Many studies have suggested that the results of animal studies can be directly equated with the equivalent developmental stage in humans, irrespective of whether analysis is performed in the fetal, prenatal, or postnatal periods (Rodier, 1980; Jacobson, 1991; Odaci et al., 2004). For example, the neonatal stage of the development of the cornu ammonis (CA) in rats is equivalent to the third trimester of CA development in humans (Dobbing, 1970; Dobbing and Sands, 1973; Rodier, 1980; Jacobson, 1991). Therefore, although there is disagreement concerning whether rat and human studies can be compared directly with one another, the findings of studies involving rats exposed to the effect of a 900-megahertz (MHz) EMF throughout pregnancy will certainly make a significant contribution to the literature concerning the same stage of development in humans.

One of the most commonly used frequencies in cell phone communications is 900 MHz (Dubreuil et al., 2002; Bas et al., 2009a,b). This study therefore investigated the effect of 900-MHz EMF. The findings from previous studies of ours suggest that 900-MHz EMF compromises the development and morphological structures of CNS tissues (Odaci et al., 2008; Odacı et al., 2013; İkinci et al., 2013; Sonmez et al., 2010). Examination of previous research on the subject shows that there have been insufficient studies using histopathological, biochemical and stereological techniques to investigate changes occurring in the hippocampus of male rats exposed to the effect of continuous 900-MHz EMF throughout all of adolescence. This study was therefore planned accordingly.

2. Materials and methods

2.1. Animals and study protocols

All procedures were compatible with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Written permission is a legal requirement for any animal experiment in Turkey. The Karadeniz Technical University (KTU) Animal Care and Ethics Committee provided formal, written confirmation that all surgical and other experimental procedures had been revised and approved by members of the KTU Ethical Committee.

Male rats were obtained from the KTU Experimental Animals Surgical Research and Application Center. On the first day of the study these were 21 days old and weighed between 28.3 and 43.9 g. Rats were kept in standard plastic laboratory cages under standard laboratory conditions at room temperature ($22 \pm 2^{\circ}C$) and humidity ($50\% \pm 10$) and in a 12 h light/12 h dark cycle. Standard rat chow (Bayramoğlu Feed and Flour Industry Trading Corporation, Erzurum, Turkey) and tap water were provided ad libitum for all animals during the experiments.

Eighteen Sprague Dawley male rats were used. These were randomly assigned into one of three equal groups, CNGr, SHMGr and EMFGr. All groups were kept in the same laboratory room, defined as the resting room, but in different cages, under the same environmental conditions, apart from during sham and EMF exposure. During sham and EMF exposure, the SHMGr and EMFGr rats were moved to another room specifically designed for EMF application, defined as the EMF exposure room. Following EMF or sham exposure, the rats were returned to the resting room. The CNGr rats received no sham or EMF exposure during the study and remained in the resting room in their cage throughout the study period. The SHMGr rats were taken to the EMF exposure room for 1 h at the same time (10:00–11:00 a.m.) every day between postnatal days 21 and 59, inclusive, but were not exposed to EMF in the EMF cage. Following sham exposure, the SHMGr rats were returned to the resting room. The EMFGr rats were then moved into the EMF exposure room, where they were exposed to 900-MHz EMF in the EMF cage for 1 h at the same time (11:00–12:00 a.m.) every day between postnatal days 21 and 59, inclusive.

2.2. Sham and EMF applications

SHMGr rats were placed inside the EMF cage every day for sham administration before the EMF group rats were placed in the cage. All SHMGr rats were placed inside the EMF cage together during sham application. Once the sham administration was complete the SHMGr rats were removed from the EMF cage and returned to their cages inside the resting room. Rat feces and other residual substances were then removed from the EMF cage using tap water and an unscented cleaning product. EMFGr rats were subsequently placed inside the EMF cage and exposed to 900–MHz EMF. All EMFGr rats were placed inside the EMF cage together during EMF application. After the EMF procedure the EMFGr rats were removed from the EMF cage and returned to their cage inside the resting room. SHMGr and EMFGr rats were permitted to move around freely inside the EMF cage during applications. The internal temperature and humidity levels in the EMF cage during EMF and sham applications were measured using a digital thermometer (YCOM KMN-303, Yu Yau Shuanghe Electron Instrument Co., Ltd. Zhejing-China). Temperature and humidity levels were similar inside the EMF cage during sham and EMF applications. Temperature inside the EMF cage was $21.9 \pm 1.09\,^{\circ}C$ during sham application compared to $21.82 \pm 1.01\,^{\circ}C$ during EMF applications. Humidity levels inside the EMF cage were $58.6\% \pm 5.72$ during sham application and $58.4\% \pm 5.41$ during EMF application.

2.3. EMF administration system

An EMF administration system was used to expose the EMFGr rats to 900-MHz EMF. The system was designed by the authors and was identical to those used in previous studies and described in detail elsewhere (Baş et al., 2013; Hancı et al., 2013, 2015; Türedi et al., 2015; Topal et al., 2015; İkinci et al., 2013; Odacı et al., 2013, 2015; Odacı and Özyılmaz, 2015). Briefly, it involved an oscillator (1218-BV, ultra-high frequency lockable oscillator, 900–2000 MHz, General Radio Company, Concord, Massachusetts, USA, Serial No. 1483), a constant electric power source (1267–B Regulated Power Supply, General Radio Company, Concord, Massachusetts, USA,

Serial No. 903) with an output power of approximately 300 mW and a frequency adjusted to 900 MHz, a half-wave dipole antenna constructed using a 1 mm (diameter) × 15 cm (length) copper plate and a Plexiglas cage (the EMF cage—30 cm × 42 cm × 50 cm). The oscillator was attached to a power source and connected to the half-wave dipole antenna via a coaxial cable. The antenna was placed in the middle of the cage, 11 cm beneath the open surface.

EMF intensity in the EMF exposure room was measured before both sham and EMF procedures. This allowed us to identify the background effects of other laboratory electrical fields and electromagnetic sources. EMF intensity was also measured during administration at nine different points both inside and outside the bottom of the EMF cage, with rats inside and with the EMF cage. The wide-range electrical field intensity meter used had a measurement interval of 100 kHz–2.5 GHz (Chauvin Arnoux CA43 Isotropic Electrical Field Intensity Meter, Paris, France). Calculations established that EMFGr rats were exposed to a mean EMF intensity of 8.4 V/m inside the jar (0.187 W/m^2). The whole-body specific absorption rate (SAR) was calculated at 0.0067 W/kg (RadHaz SAR Equivalency Calculator Version 1.0, Richard Tell Associates, Inc., Mesquite, NV).

2.4. Surgical procedures, tissue preparation and staining

At the end of the experiment all rats were weighed and sacrificed under deep anesthesia (Ketalar® 50 mg/kg, Eczacıbaşı Co., Istanbul, Turkey) by cervical dislocation on postnatal day 60. The brains were removed and weighed on a sensitive scale (KERN PCB, KERN & SOHN GmbH, Balingen, Germany). The brain hemispheres were separated, the left hemispheres being placed into tubes (Fıratmed, Istanbul, Turkey) and stored at −80 °C for biochemical analyses, while the right hemispheres were used for histopathological and stereological examinations. Once the right hemispheres had been processed through graded alcohol series and xylene, they were embedded in paraffin blocks. Hippocampal sections were prepared using a rotary microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany) with disposable metal microtome blades and a cutting angle of approximately 5° (Type N35, Feather Company, Osaka, Japan). Serial sections 30 μm in thickness were obtained from the coronal plane from the right hemisphere tissue blocks. Next, the sections were sampled using the optical dissector counting technique. This involves systematic random sampling combined with the optical dissector method (Bas et al., 2009a,b; Kaplan et al., 2012a,b; West et al., 1991). Each right hemisphere section including the hippocampus was placed onto on gelatin–formaldehyde coated slides. These were then stained with cresyl violet for stereological and histopathological assessments. A research light microscope (Olympus, BX51, Japan) was used for histopathological examination. Microphotographs were obtained using an Olympus DP 71 (Japan) camera microscope.

2.5. Estimation of total pyramidal neuron numbers

Total pyramidal neuron numbers in the CA of the hippocampus were determined from Cresyl violet-stained sections using the optical dissector method (Gundersen and Jensen, 1987; Hausdorf et al., 2008; Kaplan et al., 2012a,b; Tunc et al., 2007). The technique was performed on hippocampus sections using a stereological workstation controlled by Leica stereology software and other equipment. The system contained a computer (Intel(R) Core(TM) i7 CPU PC, Lancool PC-K56, Taiwan) with stereology analysis software (Stereo Investigator 9, Computer Assisted Stereological Toolbox-Leica) designed for the purpose by MicroBrightField (USA), a light microscope (Leica, DM4000B, Germany), a CCD camera (MicroBrightField, USA), a measuring rod measuring on the Z axis (Ludl Electronic Products, MAC6XY, Hawthorne, NY), a microcator indicator (MicroBrightField MAC 6000 System, USA), and a computer-controlled step motor whose movements along the X and Y axes on the microscope table were controlled from PC with a tray joystick (Ludl Electronic Products, MAC6XY, Hawthorne, NY).

Before embarking on the main study, we conducted a pilot study in order to determine the sampling and counting schedule. The section series were analyzed and selected in a systematic and random manner. Every sixth section was taken from the section series (section-sampling fraction = 1/6). Approximately 12–18 sections from each rat brain are reported to be adequate for estimating total pyramidal neuron numbers under the optical dissector method (Bas et al., 2009a,b; West et al., 1991). Following selection of the first section in the series, sampling was initiated from a random point between 1 and 6, with alternate sections being harvested as reserves. Coefficients of error and variation were used to confirm the efficiency of sampling and the appropriate number of sampled cells for determination of total neuron numbers (Bas et al., 2009b; Gundersen and Jensen, 1987; Odaci et al., 2010; West et al., 1991). Pyramidal neuron numbers in the hippocampal CA were calculated for all three groups. Pyramidal neurons were regarded as countable in the event that the largest pyramidal neuron nuclear profile came into focus within the virtual unbiased counting frames dispersed throughout the specified areas (Bas et al., 2009b). Total pyramidal neuron numbers were estimated according to the number of pyramidal neurons counted and the sampling probability (Bas et al., 2009b; West et al., 1991). Details of the counting and stereological procedures are given in Table 2.

2.6. Biochemical tissue analyses

The left hemispheres stored at −80 °C in a deep freezer were used for biochemical analyses. CAT, SOD, GSH and lipid peroxidation (LPO) values were calculated in these brain tissues. Decomposition of H_2O_2 in the presence of CAT enzyme activities was identified at 240 nm. CAT activity was defined as the quantity of enzyme needed to decompose 1 mmol of H_2O_2 per minute, at 25 °C and at pH 7.8. Results were expressed as mmol/min/mg of brain tissue (Aebi, 1984). Estimations of SOD enzyme activities were based on manufacture of superoxide radicals generated by xanthine and xanthine oxidase. SOD activity was measured at 560 nm by the degree of inhibition of this reaction. Activity was expressed as mmol/min/mg brain tissue (Sun et al., 1988). GSH levels in the brain tissues were measured using 5, 5′-dithiobis (2-nitrobenzoic acid) following the modifying method described by Sedlak and Lindsay (1968). The absorbance was measured at 412 nm, and tissue GSH levels were expressed as nmol/mg brain tissue. LPO levels were calculated by estimating MDA levels using the thiobarbituric acid test (Ohkawa et al., 1979). The results were expressed as nmol/mg brain tissue.

2.7. Statistical analyses

Kruskal Wallis analysis of variance was used to compare differences between group parameters at biochemical analysis. Dual comparisons between groups exhibiting significant values were evaluated using the Mann–Whitney *U* test with Bonferroni correction. During stereological analyses, the Shapiro-Wilk test was performed to evaluate the normal distribution of the variable concerned ($P > 0.05$). One-way ANOVA was then performed to test for any differences among CNGr, SHMGr and EMFGr. Finally, means were separated using Tukey HSD multiple comparisons. Variables were expressed as n (6), and mean plus standard deviation. Significance was evaluated at $P < 0.05$ for all tests. All

Table 1
The results of the biochemical analysis, mean values for total pyramidal neuron numbers, body and brain weights from CNGr, SHMGr and EMFGr rats.

Parameters	CNGr	SHMGr	EMFGr
MDA (nmol/mg tissue)	16.35 ± 1.16	17.86 ± 0.50	19.42 ± 1.12[a]
SOD (mmol/min/mg tissue)	0.34 ± 0.08	0.36 ± 0.02	0.36 ± 0.01
CAT (mmol/min/mg tissue)	93.06 ± 13.28	52.73 ± 8.31[b]	24.81 ± 5.42[c]
GSH (nmol/mg tissue)	0.70 ± 0.02	0.68 ± 0.03	0.77 ± 0.02[d]
Total pyramidal neuron number	681938.8± 93998.5	623400.7± 69642.5	502633.7± 42805.5[e]
Total body weights (end of the experiment) (gr)	238.58 ± 9.79	209.22 ± 22.10	214.7 ± 13.44
Total brain weight (gr)	1.29 ± 0.05	1.31 ± 0.10	1.30 ± 0.04

n = 6 for each group.
Data were expressed as mean ± SD. SD, standard deviation; MDA, malondialdehyde; GSH, glutathione; CAT, catalase; SOD, superoxide dismutase; CNGr, control group; SHMGr, sham group; EMFGr, EMF group.
[a] EMFGr MDA level increased compared to CNGr (p = 0.005).
[b] SHMGr CAT level decreased compared to CNGr (p = 0.004).
[c] EMFGr CAT level decreased compared to CNGr and SHMGr (p = 0.004 and p = 0.004, respectively).
[d] EMFGr GSH level increased compared to CNGr and SHMGr (p = 0.004 and p = 0.004, respectively).
[e] The total number of pyramidal neurons in the cornu ammonis of the hippocampus was significantly lower in the EMFGr rats compared to those in the CNGr and SHMGr rats (p = 0.002 and p = 0.002, respectively).

computational analyses were performed on SPSS 17.0 V statistical software (SPSS 17.0 V; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Physical examination, body and brain weights

All rats from the control (CNGr), sham (SHMGr) and EMF (EMFGr) groups were subjected to physical examination on the first and last days of EMF exposure. This revealed no skeletal anomaly or pathological findings in any rats. No significant difference was also determined among the groups in terms of rats' body or brain weights at the end of the experiment (P > 0.05) (Table 1).

3.2. Histological sections observations

Sections from CNGr (Fig. 1A and B), SHMGr (Fig. 1C) and EMFGr (Fig. 1D and E) were examined under light microscopy at varying magnifications. This revealed normal histological structures in the CA1, CA2 and CA3 regions of the hippocampus and pyramidal neurons in both CNGr and SHMGr rats. However, microscopic neuron losses were observed in hippocampal CA pyramidal neurons in EMFGr. Abundant abnormally stained cells with black/dark blue or shrunken cytoplasm at different magnifications were also observed in EMFGr compared to the other groups (Fig. 1D and E).

3.3. Pyramidal neuron numbers in the cornu ammonis

Total pyramidal neuron numbers in the CA of the hippocampus were estimated in all three groups (CNGr, SHMGr and EMFGr). A statistically significant decrease in total numbers of pyramidal neurons was determined in the EMFGr rats compared to the control (P = 0.002) and sham group rats (P = 0.002) (Table 1). No significant difference was observed in total pyramidal neuron numbers between CNGr and SHMGr (P > 0.05) (Table 1).

3.4. Biochemical analysis results

Statistical analysis of the biochemical data obtained showed that malondialdehyde (MDA) levels increased significantly in EMFGr compared to CNGr (p = 0.005). EMFGr catalase (CAT) levels decreased considerably compared to those of CNGr and SHMGr (p = 0.004 and p = 0.004, respectively), while SHMGr CAT levels were significantly lower than those of CNGr (p = 0.004). In addition, EMFGr glutathione (GSH) levels increased significantly compared to those of both CNGr and SHMGr (p = 0.004 and p = 0.004, respectively).

4. Discussion

Previous studies of ours have described the effect of 900-MHz EMF applied for varying lengths of time in both pre- and postnatal periods on pyramidal neurons in the CA region of the hippocampus of rats of either sex (Bas et al., 2009a,b; Baş et al., 2013). The common feature of those studies is that they applied stereological techniques. One previous study investigated pyramidal neuron numbers in the hippocampal CA in 16-week-old female rats following postnatal exposure to a 900-MHz EMF (1 h/day for 28 days) (Bas et al., 2009a). When the experiment was concluded, the number of pyramidal neurons in the CA was estimated based on the optical fractionator technique. The findings indicated that postnatal exposure to EMF resulted in a significant decrease in pyramidal neuron numbers in the CA in the EMF group (Bas et al., 2009a). In another study, pregnant rats were exposed to 900-MHz

Table 2
Coefficient of error and coefficient of variation of stereological analysis, optical dissector analysis data for estimation total numbers of pyramidal neurons in the cornu ammonis of the hippocampus in CNGr, SHMGr and EMFGr rats.

Stereological analysis parameters	CNGr	SHMGr	EMFGr
Dissector particle number (mean)	842	856	630
Number of sampled sections (mean)	17.83	18.16	18.16
Average section thickness (mean) (μm)	19.3	19.5	20.5
Number of steps for counting (mean)	236	193	230.6
Section-sampling fraction (ssf) (coronal)	1/6	1/6	1/6
Counting frame size (μm^2)	1.600	1.600	1.600
Area sampling fraction (asf) ($\mu m^2/\mu m^2$)	1.600/40.000	1.600/40.000	1.600/40.000
Thickness sampling fraction (tsf) (μm/μm)	10/19.3	10/19.5	10/20.5
Coefficient of error (mean)	0.06	0.06	0.07
Coefficient of variation	0.04	0.04	0.04

n = 6 for each group; CNGr, control group; SHMGr, sham group; EMFGr, EMF group

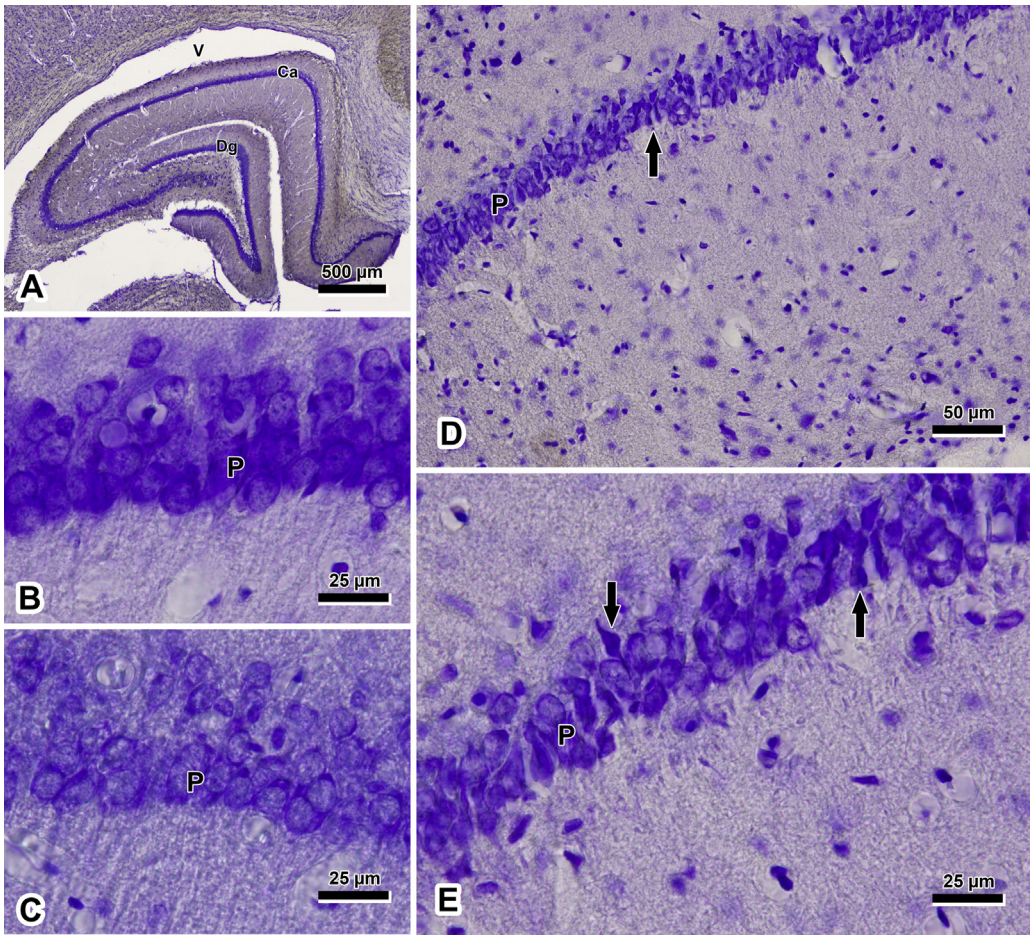

Fig. 1. Light microscopic images of CNGr (A,B), SHMGr (C) and EMFGr (D, E) hippocampal sections. Pyramidal neurons (P) in the CNGr and SHMGr hippocampi exhibited a normal appearance, while pyknotic neurons with black/dark blue cytoplasm (arrows) at different magnifications were notable in EMFGr. Cresyl violet staining. CNGr, control group; SHMGr, sham group; EMFGr, EMF group; Ca, cornu ammonis; Dg, dentate gyrus; V, ventricle; P, pyramidal neurons of cornu ammonis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

EMF during days 1–19 of gestation, inclusive. Their offspring were subsequently sacrificed for stereological analyses at the end of the 4th week (Bas et al., 2009b). Pyramidal neuron numbers in rat CA were again estimated using the optical fractionator technique. The results obtained indicated that 900-MHz EMF caused a significant reduction in total pyramidal neuron numbers in the CA in the EMF group (Bas et al., 2009b).

A more recent study by our research group reported the effect of a 900-MHz EMF applied on days 13–21, inclusive, of the prenatal period on pyramidal neuron numbers in the CA of rat pups in the postnatal period (Baş et al., 2013). In that study, experimental group pregnant rats were exposed to a 900-MHz EMF on days 13–21 of pregnancy. All female rat pups born to the pregnant rat groups were decapitated on postnatal day 32. Total numbers of pyramidal neurons in the rat pup CA were estimated using the optical dissector technique, an unbiased stereological method with a modern design. Exposure to 900-MHz EMF during prenatal days 13–21 was determined to result in a significant decrease in the number of pyramidal neurons in the CAs of the experimental group female rat pups (Baş et al., 2013). These studies indicate that, while the exact mechanism involved is uncertain, 900-MHz EMF exposure in the prenatal or postnatal periods may give rise to loss of pyramidal neurons in the CA.

The experimental design in the present study differs from those summarized above in terms of the period in which EMF was applied and the length of application. In this study, a 900-MHz EMF was applied for 1 h a day from the beginning of adolescence in rats (day 21) to the end (day 59). Despite this difference, the study resembles our previous research in terms of total cell number data and histopathological evaluation. Similar to the results of our own and other studies in the literature, pyramidal neuron numbers in the EMFGr in this study were lower than those of both SHMGr and CNGr. At histopathological examination, occasional shrunken pyramidal neurons or with dark stained cytoplasm were observed alongside pyramidal neurons with a normal appearance. The findings from this study reveal the pathological changes caused in the hippocampus by 900-MHz EMF. Other studies have reported pathological effects of EMF on living tissue. Salford et al. (2003) reported significant evidence for neuronal injury in the cortex, hippocampus, and basal ganglia of rats exposed to EMF. They described black and shrunken nerve cells, known as dark neurons, among the pyramidal neuron bands of the hippocampus (Salford et al., 2003). In that study, however, the EMF group was exposed to Global System for Mobile Communications (GSM) mobile phone EMF for 2 h.

The high MDA levels determined in EMFGr in our study are similar to those in other studies in the literature (Ilhan et al., 2004; Meral et al., 2007). In agreement with those studies, EMF in this study was confirmed to be capable of causing peroxidation in membrane lipids of nerve cells by increasing reactive oxygen species and, as a result, leading to oxidative damage in brain tissue (Ilhan et al., 2004; Meral et al., 2007). The low level of CAT in EMFGr, similar to Meral et al.'s (2007) study findings, suggested that EMF may have a depressive effect on antioxidant enzymes. We

also think that the high level of GSH, one of the antioxidant mechanisms, in EMFGr may derive from an increase in the rate of synthesis of GSH against increasing oxidative stress (Meral et al., 2007). Additionally, the cytoplasm shrinkage and darkening observed in neurons in EMFGr rats may also be interpreted as neuronal injury associated with oxidative stress. For all these reasons, it may be concluded that, based on our study findings, which are compatible with those in the previous literature, the pathological changes seen in hippocampal neurons in male rats exposed to the effect of a 900-MHz EMF throughout adolescence may be attributable to oxidative stress.

We recently investigated numbers of pyramidal neurons in the hippocampal CA in 8-week-old Sprague Dawley male rats following exposure to a 900-MHz EMF using the optical dissector counting method, a stereological technique. Our findings revealed significantly lower numbers of pyramidal neurons in the CA of EMFGr rats compared to the other groups. Abundant cells with abnormal, black or dark blue cytoplasm and shrunken morphology were also observed among the normal pyramidal neurons (Şahin et al., 2015). In the present study we also applied the stereological optical dissector counting technique for estimating pyramidal neuron numbers in the CA of the rat hippocampus. This is the most efficient and unbiased method for counting specific cells, using a virtual 3D field produced by two optical consecutive sections separated by a *t* distance (Kaplan et al., 2012a, 2012b; Sterio, 1984). This approach was first introduced by Cruz-Orive (1980) and is regarded as the first design-based stereological method in the literature (Sterio, 1984). We therefore adopted this technique for estimating pyramidal neuron numbers in this study.

Worldwide, cell phones are widely used between frequency ranges of 900 and 1800 MHz (Dubreuil et al., 2002), while in Europe they more usually employ a frequency of 900-MHz (Bas et al., 2009a,b; Dubreuil et al., 2002). This study therefore investigated the effect of 900-MHz. The threshold electric field value for 900-MHz GSM systems is approximately 10.23 V/m. This is close to the threshold value identified by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) (1998). In our study, EMFGr rats were exposed to an electric field very close to this threshold value. During the checking procedure, the intensity of the electric field was measured both inside and from beneath the cage. Our measurements show that rats were exposed to an electric field intensity approximately that of the ICNIRP (1998) threshold value.

An EMF application cage designed by the authors for use in previous EMF studies was also applied in this study (Hancı et al., 2013, 2015; Odacı et al., 2015; Odacı and Özyılmaz, 2015; Türedi et al., 2015; Topal et al., 2015). This cage was designed to be large enough for rats to move around freely inside. This was intended to minimize any potential stress deriving from immobilization. Any stress that rats might have experienced due to compression inside the cage was thus reduced to a minimum by allowing them to move around freely inside it during EMF application. However, according to our results, the SHMGr CAT level also decreased compared to CNGr. This may be due to rats being exposed to some measure of stress due to change of cage/location, even though we thought that the stress factor had been reduced to a minimum by allowing rats to move around freely within the cage, and to this stress having a depressing effect on the antioxidant enzyme. However, comparing SHMGr and EMFGr CAT values, this effect was greater in EMFGr, and the application of EMF in addition to change of cage/location may have given rise to a separate stress factor in the EMFGr rats.

Adolescence is a time of transition from childhood to adulthood (Pickles et al., 1998; Spear, 2000). Numerous ontogenetic changes occur in brain function together with neuronal changes in areas including the prefrontal cortex and hippocampus during this time. These changes are not limited to humans, but occur in a range of species, from laboratory animals (i.e. rats, mice, hamsters and rabbits) to non-human primates (Spear, 2000). Cross-species similarities in neuronal and behavioral characteristics of adolescence raise the issue of whether rats with normal developmental or pathological transition can serve as representative models of human adolescence (Spear, 2000; Odaci et al., 2004; Baş et al., 2013). Although no animal model is exactly similar in all aspects to the complex nature of human psychopathology, and although animal studies cannot be directly equated to human systems during adolescence or other stages of development, animal models have nevertheless been widely used to represent human psychopathology (Odaci et al., 2004; Baş et al., 2013).

In conclusion, our results indicate that the effect of exposure to a 900-MHz EMF throughout all stages of adolescence caused impairment in the morphology and biochemistry of the hippocampus and resulted in a decrease in hippocampus pyramidal neurons. Although the generalization to humans of results from rat studies is still the subject of debate, this study performed with the 900-MHz frequency widely applied for cell phone communication in many countries will make significant contributions to the literature concerning the effects of long-term cell phone use on living tissue. However, it is not possible on the basis of our findings to confirm the hypothesis that a 900-MHz EMF may have exactly identical effects on humans.

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